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Goetz, Werner ; Papageorgiou, Spyridon N

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Title Page

Molecular, cellular and pharmaceutical aspects of synthetic hydroxyapatite bone substitutes for oral and maxillofacial grafting

Werner Götz[1,*], Spyridon N. Papageorgiou[1,2]

[1]Department of Orthodontics, School of Dentistry, University of Bonn, Welschnonnenstr. 17, D-53111 Bonn, Germany

[2]Department of Oral Technology, School of Dentistry, University of Bonn, Welschnonnenstr. 17, D-53111 Bonn, Germany

* Address correspondence to this author at: Werner Götz, MD, PhD, Department of Orthodontics, Oral Biology Laboratory, School of Dentistry; University of Bonn, Welschnonnenstr. 17, D-53111 Bonn, Germany, Phone: +49-228-287-22116, Fax: +49-228-287-22588, email: wgoetz@uni-bonn.de

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Abstract: Bone grafts are widely used for augmentation procedures in oral and maxillofacial surgery, with autogenous bone being the gold standard. Recently, the focus of research has shifted towards synthetic bone substitutes, as no second surgery is needed and large quantities of graft can easily be provided. Within the broad range of bone substitutes, synthetic hydroxyapatite has drawn much attention, as they are considered to be biocompatible, non-immunogenic, osteoconductive and osteoinductive. Scope of this review is to summarize existing knowledge concerning the molecular, cellular and pharmaceutical aspects of synthetic bone substitutes for oral and maxillofacial grafting.

Main Text

1. INTRODUCTION

Synthetic bone substitutes belong to the family of biomaterials and represent materials for repair or regeneration of natural bone tissue. Biomaterials are defined as non-living devices used for a correct interaction or direct contact with biological systems to replace parts of living systems. Biomaterials are designated "biomimetic" if their composition, structure and properties are similar to biological materials [1-3]. Ideal biomaterial bone substitutes should fulfill certain demands like e.g. biocompatibility, integration into existing bone, promotion of healing and regeneration processes, and they should be resorbed while being substituted by new bone. They also should be able to sustain mechanical forces at the implantation site. Additionally, certain aspects of clinical demands, like low costs, sterilizability or formability should be given [4, 5]. Although autogenous bone is still considered to be the "gold standard" graft as substitute in oral and maxillofacial surgery, there are many risks and unwanted side effects in the application of autogenous bone like limited supply, additional surgical procedure, door site morbidity or risk of resorption after transplantation [6]. Synthetic bone substitutes are discussed to be a promising alternative for autogenic, but also for allogenic and xenogenic grafts [5]. Many of these substitutes are ceramics by nature, i.e. anorganic, non-metallic materials produced under high temperature by means of sintering methods. If they are specifically used for biomedical applications to repair or replace the damaged skeletal system, they are named bioceramics [3, 7]. The application of synthetic bone substitutes is increasing worldwide. Nowadays they represent an enormous market volume comprising numerous different products.

Hydroxyapatite (HA) ceramics are an important class of substitute materials. They can be of natural origin and can be derived from corals or bovine bone. The crystalline phase of synthetic hydroxyapatite (sHA) materials has a high similarity to natural HA, which is the mineral phase of bone, teeth and other hard tissues. X-ray diffraction studies have already revealed similarities between natural HA and sHA in the early 20th century. Research on and development of HA for bone augmentation has started in the earl fifties of the last century and have been increasingly used for substitution and repair as biomaterials since the 1970s, first in dentistry, later in all bone-related surgical disciplines [8]. sHAs belong to the calcium phosphate (CaP) ceramic family, which is widely used for augmentation and bone repair. This material family also includes non-sintered apatites, tricalcium phosphates (α-, β-TCPs) and mixtures, e.g. biphasic CaP consisting of HA and TCP [9-11]. In 1920 the first CaP was used in bone repair in the rabbit [12]. Nowadays, many CaP biomaterials are on the

market and have a wide range of medical and dental applications and sHA products are available in various forms, as granules, particles, powders, blocks, putties and pastes (Fig. 1), but also in injectable forms, as foams, cements and implant coatings. There are also composites available, which combine sHAs with other biomaterials, e.g. TCPs or polymers [11,13-16]. Interestingly, there is a relatively strict division of commercially available products for oral and maxillofacial surgery and for trauma and reconstructive surgery and orthopedics, although many products are identical, [5, 17]. According to last decade research data from in vitro and animal investigations and long-standing clinical experiences, it can be stated that sHAs are highly biocompatible and without immunogenic, carcinogenic or toxic side effects.

They are used for different forms of augmentation and bone defect repair in dentistry and mainly for filling defects in non-load areas in orthopedics. HAs are also used for implant coating where their bioactive properties are combined with the strength of the metal, e.g. titanium [2, 3, 18-20]. HAs are also used in other technology fields, e.g. in pharmaceutics, for liquid chromatography, catalysis carriers, as fillers for elastomers or water treatment [10]. The high protein-binding affinity and the porous character of some sHAs make them an ideal carrier for a variety of pharmacological substances in order to deliver them in many clinical applications, where there is a need of combining it with defect filling.

This review will focus on the biological behavior of pure and some modified sHAs, especially in the fields of dentistry and oral and maxillofacial surgery.

2. CHARACTERIZATION AND PREPARATION OF SYNTHETIC HA

sHAs are hydroxylated CaP salts and belong to CaP ceramics. They have a complex chemistry with Ca minerals based around P groups ($Ca_{10}(PO_4)_6(OH)_2$). They represent a hexagonal symmetric system formed by a tetrahedral arrangement of P (PO_4^{3-}) as a “skeleton”. Hydroxyl and P groups can be substituted by carbonate. This composition is similar to natural HA, although bone and dental apatites are distinguished from sHA by deficiency of Ca and incorporation of different other ions like Na, K, Mg in trace quantities under 1% [2, 3, 8, 9, 15, 21-24].

Basic knowledge of the preparation of HA is helpful for clinicians. For technical details of the production of sHAs, recent reviews are available [9, 11, 15, 21, 24-31]. In short, the two main pathways to produce sHA are high temperature processing and wet chemistry. Technically, a lot of different methods are described: solid-state processes, hydrothermal methods, mechanical-chemical synthesis, chemical synthesis including precipitation techniques, hydrolysis of calcium phosphates, mechano-chemical methods, wet precipitation, so-gel techniques,

ultrasound technologies and others. Classically, sHA ceramics are prepared by sintering [11]: Precipitation of granules or powder consisting of e.g. of pyrocalcium phosphate and $CaCO_3$ with controlled chemical purity under basic conditions and subsequent high pressure sintering at temperatures from 1000°C to 1500°C [27]. Sintering is a key step in the processing of the majority of ceramic materials. It leads to coalescence of particles and fusion of crystals. Already the combination of certain temperature, pressure and differences in the raw materials determines the properties of the final product. The composition and particle size of the powder used influences the sintering properties and composition of HA [22]. Pure HA has a stoichiometric Ca to P ratio of 1.67. With a decrease of the Ca to P ratio, Ca-deficient phases occur during the production process and other CaP ceramics like TCP, octacalcium phosphate or dicalcium phosphate anhydrate can be obtained. Several sintering techniques are available, e.g. hot pressing, microwave technologies or plasma sintering. Rising the sintering temperature increases the density and mechanical stability of the material, but reduces pore diameters and degradability. A dense HA has a lower porosity than 5% of its volume and maximum pore sizes less than 1 µm. Crystallographic investigations have also revealed a high degree of crystallinity in highly sintered sHA. The pH stability range in aqueous solutions at 25°C is between 9.5 and 12. It is stable at body temperature and under physiological pH, and it is the most thermodynamically stable CaP at pH ≥ 5.4.

The physicochemical properties of HAs such as strength or solubility, but also many aspects of bioactivity, can be modified by different methods in altering morphology, crystallite size or composition. The classical sintering process under high temperatures leads to a poor pore system with nearly no interconnectivity, as already mentioned above. Pores can be established by mixing the powder with components, which e.g. are evaporated at low temperature before sintering or are burnt out during the sintering process. For details of procedures to manufacture porous CaP scaffolds see Dorozhkin [2] and Guarino & Ambrosio [32]. Porosity is defined as the relation between the total porous volume and total volume of the material. The inner surface area is much larger in porous bodies, but the biological effects are controlled by the size of the pores and its architecture, e.g. interconnectivity [2, 3, 18]. The role of porosity in tissue response of synthetic bone substitutes is increasingly discussed under basic and clinical aspects. Porosity of bone grafts plays an important role in bone integration [33]. For the differentiation between micro- and macroporosity, different threshold values can be found in the literature. Pores smaller than 5 to 10 µm are designated as micropores. Microporosity influences the penetration of fluids, adsorption of proteins and ionic solubility but is too small for the penetration of cells. A pore size smaller than 1 µm allows interaction with proteins [3]. Microporosity is considered to be important for osteoinduction (see below), since the increased surface area could accumulate more absorbed proteins including

endogenous bone growth factors. Mesoporosity ranges between 2 and 50 µm. For macroporosity, pores are larger than 50 to 100 µm (Fig. 2). A minimum of 10-20 µm is discussed to be necessary for cellular colonization, a minimum of 50-100 µm for the ingrowing of blood vessels, fibrovascular tissue and bone tissue [3]. For bone ingrowth, pore sizes in the range of about 200 µm are recommended [34]. Karageorgiou & Kaplan [33] propose pores bigger than 300µm to enhance good capillary formation, but if there are only very small pore sizes, bone growth may only be possible on the outer surface of the material. Additionally, the interconnectivity of the porous system enhances the biological processes and tissue growth throughout the scaffold. Older investigations on tissue and vascular ingrowth into porous HA reported optimal pore diameters in the context of osteogenesis. These were between 300 and 400 µm (Ruhe et al. 2006). In general, it is accepted that macroporosity mainly favors bone ingrowth, and that connections among macropores enhance these growing processes [35]. Of course, the degree of porosity also has an impact on the mechanical properties and may thus reduce mechanical strength [35, 36].

The structure of HA allows the technical substitution of other ions without changing the symmetry significantly. The substitution e.g. of carbonate for P leads to higher solubility, the substitution of fluoride for hydroxide to a better stability, silicates (Si) replacing P to an increased bioactivity, e.g. an increase of the rate of bone apposition. The substitution with Si has been developed on the background of research 40 years ago, when it was found that Si enhances development and growth of chicks, and that it can increase bone apposition around implants. There is evidence that Si has an anabolic function in bone and connective tissue health. It facilitates bone repair, promotes osteoblast differentiation and collagen synthesis, and provides stability to biological apatite [8, 22, 37-40]. However, the biological effects of Si released from CaP material are not well understood and need to be evaluated experimentally [41]. Strontium is another element for substitution, and its beneficial effects on bone may make it become a further candidate to promote osteogenesis [42].

Nanostructured HA consists of granules lower than 100 nm size are also commercially available [43]. Nanoparticulated HAs have greater similarity to natural HA structure, and due to their larger surface area, surface roughness and wettability offer different positive biological effects: protein adsorption is increased, osteoblast and mesenchymal stem cell (MSC) adhesion, differentiation and proliferation is enhanced, osteoclast response is better [11, 44]. They can be synthesized by different methods: e.g. hydrothermal reactions in the presence of water at high pressure, the sol-gel method using a Ca-P-mix at molecular level, wet chemical methods or biomimetic deposition by nucleation and growth of HA by simulated body fluid [2, 3, 11, 12, 18, 45]. Many of these methods function with low temperatures, but disadvantages may be the long production time.

Additionally, by using modern technologies like laser radiation or silane coupling, the surface of nanostructured HAs can be modified and physical properties changed. However, the production is still complex, challenging and expensive [45]. The sol-gel technologies are newer methods for the synthesis of nanophase sHAs from Ca and P precursor combinations [11, 46]. A colloidal solution is converted into an amorphous gel, which is dried and solidified and later heated up to 600°C. This method is also useful for producing fluor-substituted HA. The synthesis of HA nanoparticles on Si gels is based on the immersion of these gels in an aqueous system containing Ca and P ions (for details see [21]). Sol-gel processes have been applied to produce Si ordered mesoporous (2-50 nm) bioceramics, which have been increasingly studied due to their high bioactivity and their ability to be loaded with molecules for controlled release of drugs or hormones [47]. Recent sHA products like NanoBone® combine nanostructuring and Si substitution: HA crystallites of 60 nm are embedded in a matrix of Si gel (Fig. 3b) and produced by sol-gel technique at temperatures below 700°C. During this process, a connection between SiO_2 and HA crystals occur, which leads to a nanoporous structure with pore size distribution between 10 to 20 nm. The granules measuring about 2-0.6 mm (Fig. 3a) have a porosity of 60 – 80% with a large inner surface in the range between 80 to 200m^2/g [48]. Size particles of about 20 nm have been proven to have the best effects on promotion of cell growth and inhibition of apoptosis [49]. The combination of nanostructuring and Si substitution revealed several positive effects on the interaction between bone and HA implants as an acceleration of the dissolution-reprecipitation process (see below) on the material surface and bone apposition [50].

All CaP ceramics are brittle and do not exhibit elastic or plastic deformation properties. HA has poor mechanical properties, low strength and toughness and lack of flexibility, which may restrict its applications in load-bearing implantation sites [2, 3, 15, 18]. The mechanical properties increase with an increasing Ca/P ratio, but decrease with increasing porosity [51]. The tensile strength for dense HA is between 79 to 106 MPa, and for porous HA around 42 MPa [13], the compressive strength of up to 900 MPa, which is higher than the strength in cortical bone. A reduced mechanical property can also be related to the environment, since wet environments reduce it. Ion substitutions can lead to changing mechanical properties, and exchange of fluoride for hydroxide leads to higher stability. Porosity has a detrimental influence on mechanical strength influencing the handling and cutting of a material by the surgeon and the behavior after implantation. Low mechanical properties can result in crumbling under stress generating micro movements, which may lead to fibrous instead of bone tissue formation [35].

3. TISSUE RESPONSES IN HEALING OF SHA BONE GRAFTS

What happens in the host tissue after the incorporation of sHA bone substitutes? Numerous *in vitro* studies, but also animal studies and histological investigations from patients after augmentation give a picture of biological phenomena occurring on the material [7, 11, 23, 52-54]. *In vitro* tests done e.g. with body fluid simulations can imitate the first steps of chemical interactions and bioactive bonding. Tests with cell cultivation using osteoblasts, osteoprogenitor cells, stem cells and others are used to investigate cellular behavior [2, 3, 7, 18, 53].

After incorporation of a graft, a healing response is normally evoked. Early stages shortly after implementation of the material in a surgical wound can be characterized as a physicochemical immersion reaction between the material and the surrounding wound bed or surrounding pre-existing bone, where blood serum originating from destroyed vessels and hematoma is the dominating body fluid. There are cascades of dissolution and reprecipitation of ions [7, 10, 15, 53, 55]. In short, Ca and P dissolute from the surface and are released into the neighborhood leading to a supersaturation of the surrounding fluid. Subsequently, they reprecipitate at the surface forming an amorphous CaP layer, which is then transformed into a carbonate containing apatite crystal layer. This process can be simulated *in vitro* by immersion of HA in simulated body fluids with an electrolyte concentration similar to serum [7, 26]. The dissolution process depends on the pH value but also the crystalline structure of the material. If there are defaults in the crystal lattice or the crystal size is reduced, dissolution will be faster. Defects at the border between HA and the surrounding pre-existing bone may also be of importance [56]. Additives in the grafting material, e.g. fluorides, can inhibit the process, Si can enhance it. Si promotes biomimetic precipitation by increasing the material's solubility and generates an electronegative surface with an exchange of Si and phosphate ions [38]. Phosphorylated proteins found in the environment of the graft, e.g. collagens or osteocalcin (OC), probably have a promoting effect on apatite formation. Anyway, in the presence of proteins the newly formed mineral phase associates with organic compounds and represents now an attractive scaffold for the attachment of cells and a chemical bond for newly formed bone. The ability to form a carbonated HA on the surface is considered to be the main aspect of CaP grafts` bioactivity [13]. The release of Ca has also positive effects on the differentiation of osteoblasts because it activates Ca-dependent protein kinases via Ca channels, leading to a higher expression of ALP, osteopontin and OC [57].

Protein adsorption via electrostatic interactions is another phase occurring on HA surfaces after impregnation by biological fluids. Theoretically, a large amount of proteins from the serum or secreted by

surrounding cells can interact with the HA surfaces [58]. Adsorption is enhanced in materials with micro pores, HA possessing small grain sizes and nanostructured materials [44, 59]. The optimum pore size for adsorption is between 20 nm and 500 μm. The nature and amount of proteins accumulated on the material surface will later determine cellular activities of the cells seeding the material. The different behavior of proteins according to material properties is due to the different properties of the amino acids [44]. In the case of the Si-substituted nanostructured HA NanoBone® it is supposed that the SiO_2 gel matrix within the graft granules is substituted by proteins during early stages of healing [48, 60]. This could be confirmed in an animal study after transplantation of this graft by dispersive X-ray spectroscopy, where the Si gel was replaced by carbohydrate macromolecules [61]. A protein rich organic matrix is formed, which can be compared with the organic matrix of natural bone. Microcrystals of the already formed carbonated HA layer together with other incorporated ions from the microenvironment associate with this organic matrix [13]. This may trigger further mineralization as a first step of bone formation. Studies concerning the healing of the nanoporous HA material NanoBone® could demonstrate the presence of typical bone proteins like OC, osteopontin (OP) or collagen type I impregnating the material´s matrix [60]. In an earlier histological study of biopsies gained after sinus lift using the porous sHA Agra®, coating of the granules by OP and bone sialoprotein (BSP) was observed [62]. This proteinaceous matrix promotes the ingrowth of connective tissue, cells or vessels as it is described for osteoconduction, but also enhances chemotaxis and differentiation of osteoprogenitor cells as it occurs in osteoinduction. Fibronectin and vitronectin are typical extracellular matrix proteins adsorbed, which favor spreading and proliferation of cells, as tests with human fetal osteoblasts have revealed [63].

Cellular interaction is the next step in the process of graft healing [2, 3, 15, 18]. It is known from *in vitro* and *in vivo* studies, that many different cell types, including fibroblasts, bone marrow stem cells (BMSC)s, osteoprogenitor cells, osteoblasts, osteoclasts, periodontal ligament cells, endothelial cells, and even osteosarcoma cells, are compatible with HA, and that they do not distinguish between natural and sHA [53]. The cellular responses of these cells can be subdivided into phases of chemoattraction, adhesion, proliferation and differentiation, and is best studied for osteoblasts and their precursors, which adhere well to HA surfaces. There are conflicting in vitro data concerning the influence of surface roughness, crystallinity, solubility or charging of HAs on cell attraction and adhesion [64]. Since cell-adhesive proteins are negatively charged, cationic HA surfaces would favor cell adhesion. Due to its higher crystallinity, HA was found to be a better substratum for BMSCs than amorphous CaP. Osteoblasts adhere well to HA surface. In general, porous HA leads to a better adhesion and enhanced differentiation of osteoblasts [2,3]. Si or zinc as components in HA increase osteoblast

attachment and proliferation [65], carbonate reduces it. Fluoride is also known to stimulate cell activity including proliferation and secretion, especially of osteoblasts [13, 53]. *In vitro* studies have already shown that substitution of Si for P ions into HA enhance osteoblast cell activity. Si substitution in sHAs also increases the dissolution of Ca and P during early stages of healing, which is an important precondition for bone formation ([56, 66]; see below). In vivo experiments in the rabbit revealed advanced bone formation [67]. Therefore, Si-based CaP grafts have already been considered as a sort of drug delivery device by releasing Si *in vivo* and influencing cell activity during osteogenesis [41].

Adhesion is mediated via integrins. When osteoblasts find a microporous structure, they form filopodia [68]. Human osteoblasts having diameters of about 20 to 30 µm, are able to migrate through very small pores which are in the range of about 2 µm [34]. After adhesion of progenitor cells or osteoblasts, proliferation, differentiation and mineralization take place. The cells produce collagen for attachment and secrete bone matrix proteins like OC, (BSP) and CaP containing mineralization granules. The influence of the material on the differentiation of osteoblasts is an emerging field of research, where cell and molecular biological aspects are of increasing importance. Only two investigations have studied the expression of different factors in osteoblasts from human bone biopsy specimens after augmentation with sHAs [69, 70]. In both studies, osteogenic factors like Bone Morphogenetic Proteins (BMPs), alkaline phosphatase, runx2 or OC were upregulated. Similar findings were observed *in vitro* after cultivation of osteoblasts on the sHA product Osbone® [71] or Biostite®, a sHA combined with equine type collagen and chondroitin sulphate [72]. OC was also upregulated in human BMSCs after cultivation on porous, S-substituted CaP [73]. Integrin binding and signaling mechanisms for adhesion and spreading as well as signaling pathways involved in differentiation are under influence of exogenous factors like Ca concentrations, or surface properties of the HA material. Intracellular signaling events regulate the stimulatory effects of the ceramic on cell function. After activation of focal adhesion kinase (FAK) a signal transduction pathway is initiated, where kinases like Ras/MAPK, ERK1/2 MAP and transcription factor complexes like AP-1 are involved [54].The activation of runx2 as the most important pro-osteogenic differentiation factor via ERK1/2 pathways plays a central role in these processes [57]. In the case that other ions are a part of the HA lattice, it may have additional effects. For example, Si released from sHA is mitogen for human osteoblast-like cells and can enhance the activity of differentiation marker and release of bone matrix proteins. An in vitro study in protein expression profiles of osteoblasts cultured on HA nanoparticles showed an upregulation of the osteogenic genes ALP and OC, and the regulation of several genes involved in Ca metabolism [74]. Investigations in the rabbit revealed a very early bone ingrowth of Si substituted porous HA

implants in contrast to stoichiometric HA by using a Si level of 0.8wt% [67]. It can be concluded from in vitro and in vivo studies, that Si mainly influences early events of bone regeneration by HA scaffolds.

The process of new blood vessel formation and vascularization during the healing of bone grafts should not be underestimated. Blood supply is a critical factor in reconstructing bone defects with all substitutes used [75]. However, from a biological point of view, angiogenesis, i.e. the *de novo* formation of blood vessels, and vasculogenesis, the sprouting of vessels from pre-existing vessels, should be differentiated. Histological studies observing the healing process of synthetic bone grafts indicate, that probably only vasculogenesis can be observed. Bone graft substitutes have no cells in the graft and need more time for vascularization. For the development of an efficient neovascularization, pore sizes between 150 and 500µm, and a cross-talk between the pre-existing bone vasculature, osteoprogenitor and endothelial cells are required [76]. Angiogenesis and osteogenesis are closely coupled. This coupling is tightly regulated by an auto- and paracrine network of different factors, e.g. Vascular Endothelial Growth Factors (VEGFs). Kilian *et al*. [77] could demonstrate that VEGFs and VEGF receptors are upregulated in critical size bone defects in the mini pig with implantation of nanosized HAs. VEGF was also found in human biopsies after augmentation with different sHAs [60, 70]. VEGFs are secreted by osteoblasts and induce the differentiation of endothelial progenitor cells. Angiogenetic transcription factors are upregulated and stabilized under hypoxic conditions, which may prevail during wound healing. The occurrence of hypoxia inducible factor (HIF-1α) during healing of a nanosized sHA is an indicator for these conditions [78]. However, other exogenous factors can influence the process of vasculogenesis, like the state of the pre-existing vascularization of the host tissue, aspects of the surgical procedure in augmentation, or mechanical interactions between the environment and the graft. The vascularization of the host should also be taken into account under clinical aspects, because there are many patient related systemic aspects leading to reduced circulation: smoking, old age, systemic diseases like diabetes, radiation and others. Histologically, differences in the way of vascularization of bone substitutes can be observed: While in case of some substitutes, vessels come in close contact to the graft, a vascular perforation and even branching of new vessel into and inside the graft has been observed in others. These vessels may transport osteoblastic precursor cells into HA granules, as already discussed for the vessel ingrowth into the nanostructured bone substitute NanoBone® [60, 78]. Positive effects of HA on angiogenesis are supported by *in vitro* studies showing a high biocompatibility of microvascular endothelial cell son HA nanocrystals [79].

For porous HA, an ingrowth of fibrovascular tissue into the center of the graft has been described, followed by bone apposition, which begins along the walls of the material [80] (Fig. 4). Appositional bone formation

around or in HA grafts occurs similarly to intramembraneous osteogenesis of natural bone. After deposition of osteoid by osteoblasts and subsequent mineralization, fibrous or woven bone appears, which later is remodeled into mature lamellar bone tissue [80]. Woven bone should be remodeled into mature lamellar bone and should show similar stability like the neighboring autochthonous bone. In case of slow resorbing HAs these processes may take years. However, histological investigations of bone substitutes which had been integrated for several years can undergo late remodeling or revitalization [81]. Non-resorbed HA residues can remain in close contact with the newly formed bone, and can even be incorporated into the bone tissue ("osteocoalescence" [82]). They can be bonded with an interface to the newly formed bone [13]. These intimate bonding zones resemble cemental lines of natural bone and represent dense zones containing a mineralized organic meshwork with large HA crystals, containing carbonate and highly mineralized collagen. The interfacial strength is great and fractures occur either in the HA material or the surrounding new bone, but not at these interfaces [26, 53]. Mechanical stress leads to cohesive failure of the material or bone, but not at the interface [13].

Probably, HA may have surface areas that meet electrical and spatial requirements for primary bone bonding allowing this dense attachment between the graft and surrounding bone without any ingrowth into the material [83]. In later stages of healing, the newly formed bone around HA grafts or the non-degraded material itself may have an intimate bonding creating a strong interface.

During the healing of HA grafts, inflammatory reactions do not belong to the normal healing process [84]. Although slight inflammatory reactions are believed to occur being part of a mild foreign body process. Due to its similarity to biological apatites, sHAs may not be recognized as foreign material. Proteins adsorbed from the environment can activate macrophages, but no adaptive immune response may occur when there is no pathogen invasion due to the implantation of the graft material [85]. Inflammation should always be due to other reasons related to the surgical technique or local or systemic aspects from the host tissue and patient, e.g. wound inflammation, migration of HA granules etc. [2, 3, 18]. Mutagenicity and carcinogenicity have been tested for different commercially available HAs. They did not show any indications for these unwanted side effects [86].

The behavior of bone substitutes in patients impaired by local or systemic diseases has yet not been well investigated. Physiologically, age-related bone loss or insufficient vascularization can reduce all biological aspects of graft healing, whether by osteconduction or osteoinduction. In one recent study it was confirmed by histological and histomorphometrical examinations that after applying a nanocrystalline sHA for sinus lift, no age-dependent differences between a younger and older aged patient group could be documented [87]. It was shown in another recent clinical and histological study, that nanocrystalline sHA proved to be useful

augmentation material for a sinus lift in patients with previous oral cancer [88]. It is also critical to determine whether bone regenerative approaches with HA grafts are effective for healing craniofacial bone defects challenged by therapeutic radiation [76].

4. OSTEOCONDUCTION OR OSTEOINDUCTION?

Bioinert materials are not able to stimulate bone formation or have minimal response from the host tissue, e.g. fibrous layer formation [8]. Substitutes should be at least bioactive, which means being able to establish a direct, adherent bonding and interface with the host bone and to stimulate and promote osteogenesis [83]. Traditionally, the properties of bone substitute graft osteoactivities have been classified in three main categories: Osteogenesis as the ability for intrinsic bone formation. This means that the material must contain viable osteoblasts to be able to form bone or to differentiate into osteogenic cells, a property which is fulfilled only by autogenous grafts. Osteoconduction is the ability to promote and support healing by enhancing osteogenesis on its surfaces and to serve as a scaffold or template for the growth of new bone. The graft serves as a place holder and guide bar allowing ingrowth of cells, vessels and connective tissue. Newly formed bone is deposited on the surface of the material, which should be degraded by time. This sort of bone apposition has also been named "creeping substitution" (Fig. 4). Osteoinduction is a process, where bone formation is induced by activating growth factors, mainly BMPs, and the recruitment of endogenous stem or progenitor cells, which differentiate towards the osteoblastic lineage. Additionally, it should be able to induce ectopic or heterotopic osteogenesis, e.g. subcutaneously or intramuscularly, in the animal experiment [9, 15, 64, 89-91]. The term "osteopromotion" is often used to characterize enhancement of osteoinduction without having osteoinductive properties.

There is an ongoing discussion about the osteoinductivity of synthetic bone substitutes, especially CaP [3, 53, 64, 92, 93]. *In vitro*, CaP can exert positive osteoinductive effects when MSCs or adipose stem cells are cultured, albeit in the presence of osteogenic supplements as dexamethasone or ascorbic acid. However, other calcium phosphates than HA like TCP or HA/TCP combinations have higher osteoinductive potentials. Some animal studies have shown that ectopic bone formation can be induced, and that growth factors, such as BMPs, and differentiating osteoprogenitor cells can be found at the implantation site. Obviously, the ability for ectopic bone formation is species-dependent. While osteoinduction by HAs does not function e.g. in rodents, osteoinduction for sHAs is possible in dog [89, 93-95]. Histological investigations in human biopsies from augmented regions have shown similar findings. The reason for this ability is unclear, whether the physicochemical or surface structure of some HAs is responsible for these effects or whether growth factors or

other inducing molecules are adsorbed by the material from the environment. Porosity, especially microporosity, and interconnectivity with the resulting increase of the inner surface may play an important role [95, 96]. BMPs can be trapped, especially when the microporosity of the material allows influx or absorption of these molecules. The ceramic may also potentiate the activity of BMPs by binding the protein and presenting it to target cells. Former studies have already described an upregulation of BMPs by CaP bone grafts [97]. BMP-2 has been detected immunohistochemically in the host region around the grafted particles during early healing of a nanostructured sHA [60], indicating an absorption of these factors or activating endogenous BMP from the environment. However, BMPs may not always be necessary for osteoinductive processes if the biomaterials´ physicochemical functions are sufficient [94]. Low oxygen tension may play an important role in the graft´s environment. Pericytes located in the vessel walls are differentiating into osteoblast under low oxygen [2, 3, 18, 98]. Several working groups believe that HA have intrinsic osteoinductivity, but that the potency is depending on chemical properties, geometry, porosity, surface concavity, which allow the entrapment of osteogenic growth factors and osteoprogenitor cells [93]. Future work should concentrate on the cellular and molecular mechanisms which are defining the physicochemical and structural characteristics with osteoinductive properties. It should be clearly demonstrated *in vivo* whether osteoprogenitor cells or mesenchymal stem cells are attracted and differentiate to osteoblasts around or in the HA graft. In the above mentioned study of Götz *et al.* [60], runx-2 immunoreactive osteoprogenitor cells could be identified, which were even able to penetrate the HA graft matrix or have been transported to central parts of the graft granules by vessels.

Engineering osteoinductivity for synthetic HA would therefore be a challenging approach for improvement of bone regeneration by HAs [99]. However, it has to be questioned, if osteoinductivity will achieve a clear benefit for the healing of bone ceramics clinically, and if the clinical success and the patient's comfort will be the same for both processes. Osteoinductivity could be an advantage in cases of large defects, or when the host tissue is compromised with low regenerative capacities. Applying ceramics with good intrinsic osteoinductivity also does not need to be substituted with other factors enhancing osteogenesis. This may save costs. Anyway, osteoinductive processes depend on the presence of stem or progenitor cells in the environment.

5. DEGRADATION

Degradation behavior of bone substitutes is of great clinical interest in terms of predictability and control. Unfortunately, degradation is very complex, since it is influenced by many factors such as physicochemical characteristics of the material to be degraded, and biological aspects and individual factors of the host [2, 3, 18,

53, 100]. A constant decomposition and concomitant substitution with natural bone tissue is desirable in many clinical circumstances. However, in some cases, a longer place-holding function with only slow degradation may be preferable. A very fast degradation may negatively influence osteoconductivity, because connective tissue ingrowth into empty spaces may be faster than osteogenesis. Degradation can also be reinforced by mechanical stress at the implantation site [82].

There is a general agreement that synthetic HA belongs to the group of substitutes with a slow degradation rate [11, 13]. Degradation of non-porous, dense and phase pure sintered HA will last for months and even up to years, but porous HA or HA powder will degrade faster. Older investigations described a resorption rate of 5 to 15% per year after orthopedic application [101]. Degradation of sHA is hard to control and predict due to a great number of influencing factors and individual variations among patients [102].

Like it is the case for nearly bone substitutes, chemical liquid dissolution with lower pH, i.e. biodegradation, as well as cellular resorption, also called bioabsorption, have been described [53, 100]. In general, the dissolution rate is inversely proportional to the CaP ratio, purity and crystalline size and directly related to the porosity and surface area. Lattice defects in the material can also be involved in the dissolution process [82]. Dissolution can be tested *in vitro* by suspending the material in acidic buffer. Tests with different CaP materials revealed degradation potency in the following order: ß-TCP, non-sintered bovine bone apatite, sintered bone apatite, coralline HA, sHA [2, 3, 18, 26]. The relationship between the acellular dissolution and the cellular degradation of synthetic HA *in vivo* is not clear. Are there specific circumstances favoring one of them, or do they appear in a temporal or spatial relation? Zarbo *et al*. [103] discussed if the nature of the calcium phosphate, e.g. its solubility, plays an important role. TCP, which is more soluble than HA for example, will undergo dissolution rather than cellular degradation. It is clear that cells with phagocytic activity are candidates for the cellular HA degradation. These are monocytes, macrophages, osteoclasts and osteoclast-related cells like giant cells. *In vivo*, dense HA is not resorbed until its particle size is suitable for phagocytosis. The resorption of HA by osteoclasts is similar to natural bone: Osteoclasts form by the fusion of monocytic cells from the bloodstream, become activated and thus colonize the graft. They adhere with actin rings, form a sealing zone, create an acidic environment, form resorption pits and phagocytes [53, 83]. The reason why osteoclasts prefer settling on HA material can be seen on the proteinaceous character of graft granules mimicking bone matrix ([60]; see above). Especially, ostepontin, which can be detected as a molecule within this matrix, favors adhesion and differentiation of osteoclasts. Histological studies on human biopsies from regions augmented with sHA give a classical picture of osteoclast formation and activity [60]. *In vitro* studies have revealed that the

behavior of osteoclasts seems to be different on bone substitutes [104]: While on ß-TCP they form deep lacunae and the resorption on HA is only superficial. *In vitro* colonization investigations have revealed, that a nanoscale structure of HA can increase the osteoclastic response [44, 105] (Fig. 5). Furthermore, the solubility of a HA can determine osteoclast activities by controlling the increase of Ca ions dissolved [83]. Substitution of HA with fluoride can inhibit osteoclastic resorption [13], while on Si-substituted sHAs osteoclast activities are enhanced, which e.g. can be demonstrated by production of larger and more numerous resorption lacunae [40, 106, 107]. Active osteoclasts seeded on nanoparticular sHA *in vitro* release Ca ions into the culture medium [108]. It is still discussed, whether all HA resorbing multinucleated cells resemble osteoclasts, or whether a subpopulation consists of foreign body giant cells. In general, osteoclasts can be characterized on tissue sections by staining the enzyme tartrate resistant acid phosphatase (TRAP). However, both cell types have a number of common factors [109], which complicate further studies. Immunohistochemistry using ED1 as a marker of the mononuclear phagocyte family showed that macrophages as osteoclast precursors appear around vessels during the healing of a nanophase HA [60]. Considering histological observations, it seems obvious that also macrophages are involved in the phagocytosis of sHA. They probably phagocyte very small particles below the size of 10 µm [84, 85]. However, the relationship between them and osteoclasts in the resorption process is not clear. It is also unknown whether factors like cytokines and growth factors secreted by macrophages participate in proliferation and differentiation processes of cells around grafts [85].

As already mentioned, bone apposition and concomitant degradation of the graft material going “hand in hand” would be a desirable process allowing a “smooth” transition of the substitute. Histological and immunohistochemical studies from human biopsies and animal studies [60, 110] on the healing of the nanostructured HA NanoBone®, show a picture of “compartimentaliaztion” of the grafts, due to the observation of a side with TRAP-positive osteoclasts and an osteogenic area with osteoblasts and bone apposition around the same graft granule. This demonstrates a possible integration of the bone-grafting material into physiological remodeling processes of the host. Further studies should elucidate which molecular factors control this process.

In the context of degradation, no clear evidence for cytotoxicity, carcinogenicity or genotoxicity of sHA particles is given. Degradation products like Ca or P ions could be transferred from the phagocyting cells to the blood. However, no abnormal levels of these ions could be observed in the patients´ serum, urine or organs [111]. Also, the biocompatibility of Si-substituted HAs has been tested [112]. However, the biosafety of HA nanoparticles remains controversial. A generation of wear particles from nanostructured HA may be possible, which are released and transported into surrounding tissues, e.g. lymph nodes. These particles may be locally

cytotoxic to osteoblasts or can aggregate intravascularly [11]. Especially in the context of the discussion about toxicology and biocompatibility of nanomaterials, there is concern about local toxic or inflammatory systemic side effects of nano grafts [43, 113], although there are no evidence based data on the toxicity of nanostructured sHAs at the moment.

6. Outlook

As a basis for development of improved sHAs it is necessary to fully clarify the biological processes of integration, healing and degradation of existing products, to study biocompatibility and probable local and systemic side effects. It should be taken into account that HA grafts used in the orofacial region are implanted into a special bony region with environmental differences and biological peculiarities, which are not similar to those of the extracranial skeleton [76]. Therefore, research should consider these special circumstances and e.g. carry out *in vitro* studies with osteoblasts from jaw bone. The effects of the architecture, which means differing properties like porosities, interconnectivity, surface chemistry etc., on the success of clinical applications also require more studies. Additionally, basic mechanisms of osteogenesis, degradation of HA grafts in compromised patients and possible influences of systemic diseases should be elucidated. In this context, further studying adsorption mechanisms, signaling molecules involved in osteoconductivity and osteoinductivity as well as cellular and molecular aspects of degradation and resorption will be of eminent importance [2, 3, 18]. The engineering of intrinsic osteoinductive HAs is considered to be a future alternative to autogenous or allogenous grafts in order to accelerate bone formation or to use these materials for implant coating [26]. Also the improvement of the mechanical performance of the existing HA ceramics will be necessary [8]. For the clinician, an easy handling, shaping and application will be important. In the context of bone tissue engineering, the development of complex 3D scaffolds made from HA materials is of great interest [14, 76].

CaP ceramics did not play an outstanding role as scaffolds or carriers in bone tissue engineering research in the last decade [114]. However, experimental studies on the biofunctionalization of sHAs have already been performed and focus on the improvement of coatings to deliver biological agents and to develop smart materials [8]. Of course, nanophase ceramics are a promising type of bone substitute for these efforts [115, 116]. As already mentioned, sHAs and especially nano-structured HAs are excellent drug carriers, and may not only be used to carry osteogenic or angiogenic growth factors, but also drugs, e.g. with antibacterial effects [11, 14, 82, 117-120]. For discussion on antibiotic release from CaP materials see also the article of Prados-Frutos et al. in this issue. Association of CaP ceramics with BMPs could be a way for a controlled release of these growth

factors, which are intensively studied in many experimental and clinical fields of bone regeneration [114, 119, 121, 122]. Further fields of application concern local delivery of anti-cancer drugs. The functionalization of HA nanocrystals with platinum-bisphosphonate complexes to treat bone tumors has been successfully tested in vitro [123].

The combination of HAs with cells, especially stem cells, will be another challenging field of investigation [76, 124]. The use of MSCs or induced pluripotent stem cells seeded on suitable scaffolds appears to be a potential approach in bone defect therapies. The proliferation and differentiation of these cells into an osteoblastic phenotype has been shown [120, 125]. When bone substitutes will be seeded with cells, different problems like seeding efficiency, density or predifferentiation of the cells have to overcome. However, only very few preclinical studies involving human MSC transplantation have been done using HA grafts [124, 126]. In a clinical study, 10 patients were treated with autogenous BMSCs seeded on a porous sHA which were implanted into intraoral bone defects. According to histological findings in biopsies taken 4 months after reconstructing, bone formation could be demonstrated in 3 patients. Only in one patient osteogenesis seemed to be induced by the construct [127]. Future strategies for bone tissue engineering will deal with gene delivery, e.g. of microRNAs, which are transcriptional regulators. Their roles in controlling osteogenesis and bone metabolism are currently under investigation [128]. However, for all this new technologies to create molecularly tailored bioceramics, clinical translation will be a complicating step [129, 130].

LIST OF ABBREVATIONS

BMP = bone morphogenetic protein

BMSC = bone marrow stem cell

BSP = bone sialoprotein

Ca = calcium

CaP = calcium phosphate

HA = hydroxyapatite

HIF = hypoxia inducible factor

MSC = mesenchymal stem cell

OC = osteocalcin

OP = osteopontin

P = phosphate

sHA = synthetic hydroxyapatite

Si = silicate, silicium

TCP = tricalcium phosphate

TRAP = tartrate resistant acid phosphatase

VEGF = vascular endothelial growth factor

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Figure Legends

Figure 1: Examples of commercially available sHA forms as granules and blocks (NanoBone®)

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Figure 2: Scanning electron microscopy of a sHA with interconnecting macropore sizes between 100 and 250 µm (Osbone®/IngeniOs®); x50

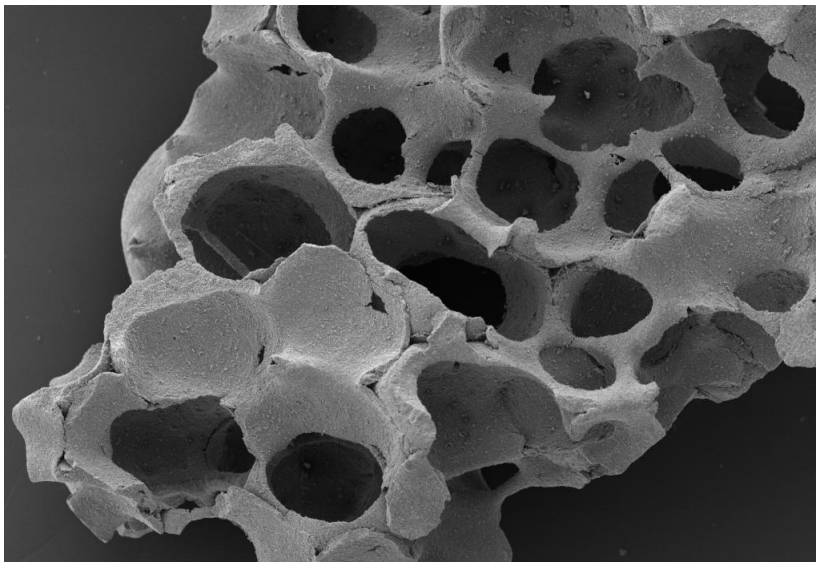

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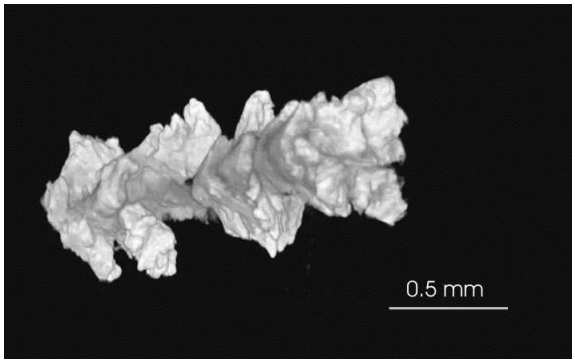
Figure 3: Ultrastructure of a Si-substituted sHA (NanoBone®): **a**. Granule, Micro-CT; **b**. HA crystals embedded in silica gel; transmission electron micrograph

(a)



(b)

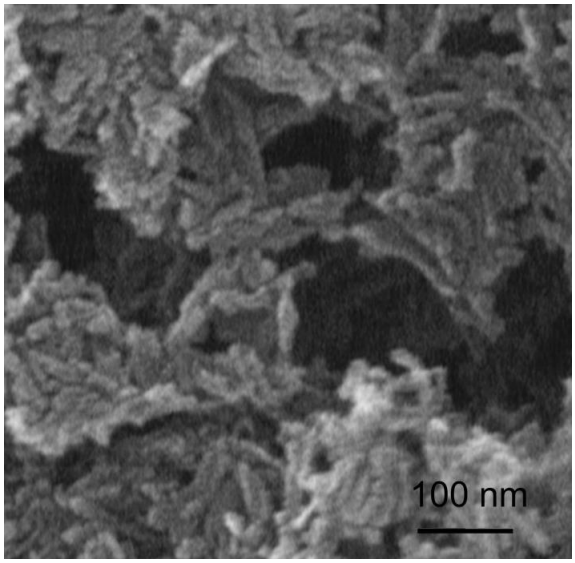


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Figure 4: Histological appearance of osteogenesis (asterisks) around sHA granules (g); biopsy, augmentation with NanoBone®, sinuslift, 6 months healing; hematoxylin eosin staining, x10

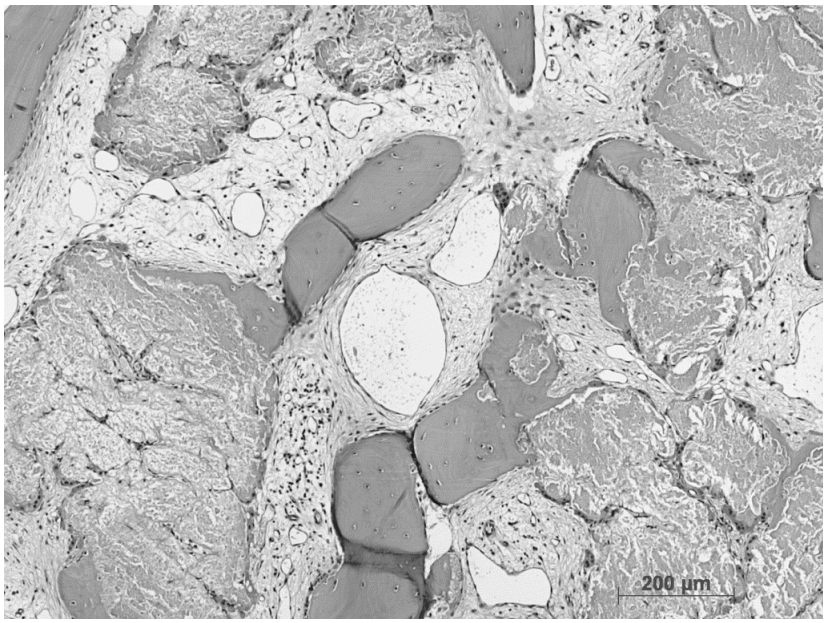

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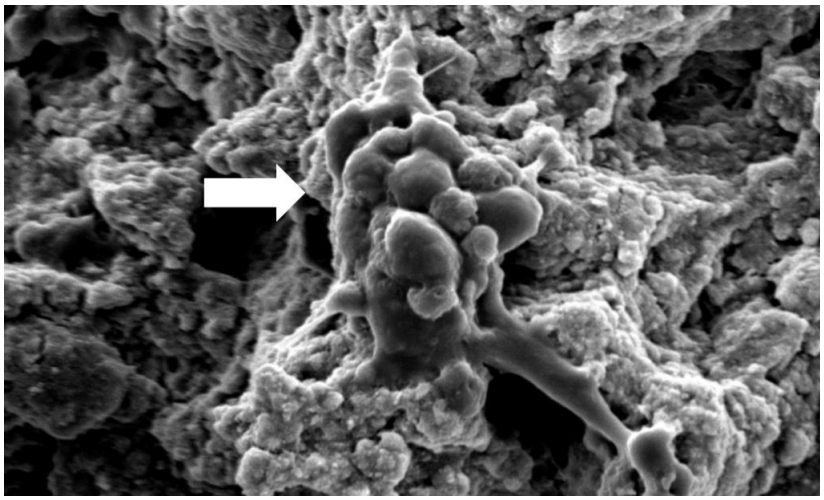
Figure 5: Osteoclast (white arrow) with multiple bulging nuclei seeded on a sHA material (NanoBone®); scanning electron microscopy, x2000



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